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Modulation of erythrocyte hemorheological properties by band 3 phosphorylation and dephosphorylation

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Abstract. The objective of the present work was to study the effects of erythrocyte proteins phosphorylation in erythrocyte aggregation and deformability. Human whole blood samples were incubated *in vitro* in absent and in presence of the phosphorylation/dephosphorilation band 3 inhibitors and also with adenylyl cyclase, guanylate cyclase and PI3K inhibitors and the erythrocyte aggregation index (EAI) and deformability were assayed. The results show that when band 3 is phosphorylated in presence of a PTP inhibitor an increase in erythrocyte aggregation index is observed ($p < 0.0001$). A partial dephosphorylation band 3 state, induced by PTK inhibitors, show a decrease in the erythrocyte aggregation index ($p < 0.002$). However both manipulated states induced lower EAI values than blood samples aliquots controls. The guanylate cyclase and PI3-K inhibitors significantly decrease the erythrocyte aggregation index in relation with the control blood samples. Erythrocyte deformability in presence of all the inhibitors did not showed significant changes. PTP and PI3-K inhibitors showed a significantly increase in the plasma potassium concentrations not associated with EAI values. Methehemoglobin levels were increased significantly when guanylate cyclase inhibitor is present in the blood samples. In conclusion, the results suggest that erythrocyte aggregation index is dependent of the phosphorylated/dephosphorylated state of band 3.

Keywords: Band 3, erythrocyte, phosphorylation, dephosphorylation, protein tyrosine kinase, protein tyrosine phosphatase

1. Introduction

The red blood cells (RBCs) tendency to aggregate is destabilised by the blood flow and also by the repulsive forces coming from the outer membrane molecule charges [1,2,17,25,37]. The erythrocytes are brought together forming "rouleaux" that are mediate by plasma proteins among which the fibrinogen molecule receives the major preponderance [36].

An increase in the fibrinogen is observed in those pathologies associated with inflammatory conditions such as cardiovascular diseases [1,19,24,25], being the fibrinogen considered as a cardiovascular risk factor [4,5,14,18]. For example in hypertension and in atherosclerosis the increase in erythrocyte aggregation could be attributed to an increase in plasma fibrinogen concentration [10,30,41,42].

There are several *in vitro* studies where maintaining constant plasma fibrinogen concentration the red blood cells aggregation tendency changes in presence of effectors namely β-estradiol, buflomedil,

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epinephrine, ethanol, flunarizine, acetylcholine, spermineNONOate, calcium and choline [3,4,11,12,26, 31–33,39,45].

Erythrocyte hyper aggregation and deformability impairment could together increase blood viscosity either hindering blood flow in large vessels or occluding it in the microvessels [16,18,28,34,35].

Several approaches could be made to understand the RBCs aggregation changes both *in vivo* and *in vitro*. For example we have observed that applying fluorescence methodology β-estradiol binds to fibrinogen with changes in the protein conformational states [38]. These results could tell us that some fibrinogen molecules were not in the optimal conditions to bridge RBCs, favouring the decrease of erythrocyte aggregation as has been verified [11].

Looking inside the erythrocyte there are evidences that cytoskeleton protein phosphorylation modify the membrane stability [40] and that tyrosine phosphorylation of protein band 3 inhibits glycolytic enzymes binding [29] but facilitate the efflux of nitric oxide [44].

Nitric oxide synthase inhibitors and guanylate cyclase inhibition reduced erythrocyte deformability, suggesting that NO is a determinant of RBC mechanical behaviour [22].

It was observed by us that adding acetylcholine to erythrocyte suspensions the nitric oxide (NO) mobilization and the levels of nitrites and nitrates are increase [6]. We have raised the hypothesis that the phosphorylation/dephosphorylation of band 3 could be responsible for the signal transduction mechanism induced by acetylcholine [6], which could explain the RBC hemorheological parameters behaviour previously obtained [33].

In this sense the aim of this work was to verify if the modulation of the phosphorylation degree of band 3 influence the human erythrocyte hemorheological properties. In particular, the *in vitro* effects of band 3 inhibitors of phosphorylation/ dephosphorylation and adenylyl cyclase, guanylate cyclase and PI3K inhibitors were evaluated.

2. Material and methods

2.1. Materials

General reagents were provided by Sigma-Aldrich Co. For protein tyrosine kinase (PTK) inhibition Syk, a PTK p72syk inhibitor (Calbiochem, Merck, Darmstadt, Germany), Aminogenistein (AMGT), a PTK p53/56lyn inhibitor (Calbiochem, Merck, Darmstadt, Germany) and DMPQ (Calbiochem, Merck, Darmstadt, Germany), a potent and selective inhibitor of human vascular beta-type platelet derived growth factor receptor tyrosine kinase were used. Calpeptin (Calp) a calpain inhibitor, from Calbiochem, Merck, Darmstadt, Germany was used for PTP inhibition. Guanylate cyclase inhibition was achieved with Ly-83583 (Ly) also from Calbiochem, Merck, Darmstadt, Germany. Wortmannin (Wort), a PI3-K inhibitor and MDL hydrochloride (MDL) an adenylyl cyclase inhibitor were purchased from Sigma-Aldrich Co. Inhibitors were prepared as recommended in the information sheet of the products: Wort, AMGT, Ly, Syk and Calp were prepared in a DMSO solution at 10^{-3} M concentration; DMPQ and MDL were prepared in distilled water at the same concentration.

2.2. Blood sampling and incubation procedure

Blood was supplied, according protocol, by the Portuguese Institute of Blood, Lisbon. Blood samples were collected into tubes with lithium heparin (17 IU/ml) as anticoagulant. Total blood was divided into two aliquots of 1 ml each and centrifuged at 11 000 rcf (Biofuge 15 centrifuge, Heraeus) during 1

minute at room temperature. Then 10 μl of plasma were replaced by the same volume of either physiological serum or inhibitor, so that the final concentration of the inhibitor in the whole plasma was 10^{-5} M. Besides other concentrations (5×10^{-5}, 10^{-6} and 5×10^{-6} M) were tested, no significant alterations were observed with these concentrations in relation to 10^{-5} M. Then the blood sample was homogenised by gently inversion and erythrocytes aggregation and incubated for 15 minutes, then deformability, pH, oxygen tension at half saturation of blood (p50), ions (sodium, potassium and calcium), hemoglobin (Hb), oxyhemoglobin (O_2Hb), methemoglobin (MetHb) and hydrogen carbonate (HCO_3^-) concentrations were determined.

2.3. Erythrocyte aggregation index

Erythrocyte aggregation was determined using the MA1 aggregometer from Myrenne GMBH (Roetgen, Germany). The MA1 aggregometer consists of a rotating cone plate chamber, which disperses the sample by high shear rate of 600 s^{-1}, and a photometer that determines the extent of aggregation. The intensity of light, exerted by a light emitting diode is measured after transmission through the blood sample. The aggregation was determined in stasis for 10 seconds, after dispersion of the blood sample [25].

2.4. Erythrocyte deformability

The erythrocyte deformability for different shear stress (0.30, 0.60, 1.20, 3.00, 12.00, 30.00 and 60.00 Pa) was determined by using the Rheodyn SSD shear stress diffractometer from Myrenne GMBH (Roentgen, Germany) and erythrocyte deformability is expressed as the elongation index (EI) in percentage. The Rheodyn SSD diffractometer determines RBC deformability by simulating the shear forces exerted by the blood flow and vascular walls on the erythrocytes [8]. Erythrocytes are suspended in a viscous medium and placed between a rotating optical disk and a stationary disk. A well-defined shear force is exerted upon the suspension which forces the erythrocytes to deform to ellipsoids and align with the fluid shear stresses. If a laser beam is allowed to pass through the erythrocyte suspension a diffraction pattern appears on the opposite end. That diffraction pattern will be circular with resting erythrocytes, but becomes elliptical when the erythrocytes are deformed by shear. The light intensity of the diffraction pattern are measured at two different points (A and B), equidistant from the center of the image. The erythrocyte elongation index (EEI), in percentage, is obtained according the following formula:

$$EEI(\%) = \frac{A - B}{A + B} \times 100.$$

2.5. K^+, Na^+, Ca^{2+} and HCO_3^- plasma concentrations, pH, p50, Hb, O_2Hb and MetHb determinations

After the additions of the inhibitors Hb, O_2Hb and MetHb were determined with the Osm3 hemoximeter from Radiometer. pH, p50, K^+, Na^+, Ca^{2+} and HCO_3^- concentrations in blood were determined with the ABL505 Blood Gas-Electrolyte System from Radiometer (Copenhagen, Demark).

2.6. Statistical methods

Data are presented as means $\pm$ SD. The differences between the mean values were evaluated by using Student's t test for paired samples. A p value less than 0.05 was considered statistically significant.

3. Results

3.1. Effects on erythrocyte aggregation and deformability

The EAI values changes significantly when blood samples were incubated with phosphorylation and dephosphorylation inhibitors in relation to the control value of 15.94 ± 3.6 (nd). Comparing an almost dephosphorylation state of band 3 (AMGT and DMPQ) with a totally phosphorylated state (Calp) there is a significant difference in EAI ($p < 0.04$). However both manipulated *in vitro* phosphorylated and desphosphorylated states induce lower EAI values when compared with the control aliquot (Fig. 1).

The presence of the guanylate cyclase inhibitor (Ly) and PI3-K inhibitor (Wort) in the incubated aliquots, decrease significantly the EAI, $p < 0.0002$ and $p < 0.001$, respectively, when compared with the control. Adenylyl cyclase inhibitor (MDL) did not significantly change erythrocyte aggregation in relation to the control aliquot (Fig. 2).

Figures 3 and 4 showed that the values of erythrocytes elongation indexes measured at different shear stresses did not significantly change with the presence of any of the used inhibitors in the blood samples aliquots when comparing with the control aliquot.

3.2. Effects on sodium, potassium, calcium and hydrogen carbonate concentrations

In all blood samples aliquots there were no significant variations on the plasma calcium, sodium or hydrogen carbonate concentrations in presence of any of the inhibitors used in the study (Table 1) when compared with the control aliquot. Highly increased values were obtained in presence of the PTP inhibitor (Calp) ($p < 0.0001$) and also in the presence of the PI3-K inhibitor (Wort) ($p < 0.004$) for K^+ plasma concentration in relation to the control aliquot no other statistical differences were obtained when the remainder inhibitors were added to blood samples aliquots in relation to the control one (Figs 5 and 6). Comparing those potassium concentrations values with the EAI values obtained we did not obtained significant correlation between the two parameters, meaning that the aggregation alterations are not plasma K^+ concentration dependent (Fig. 7).

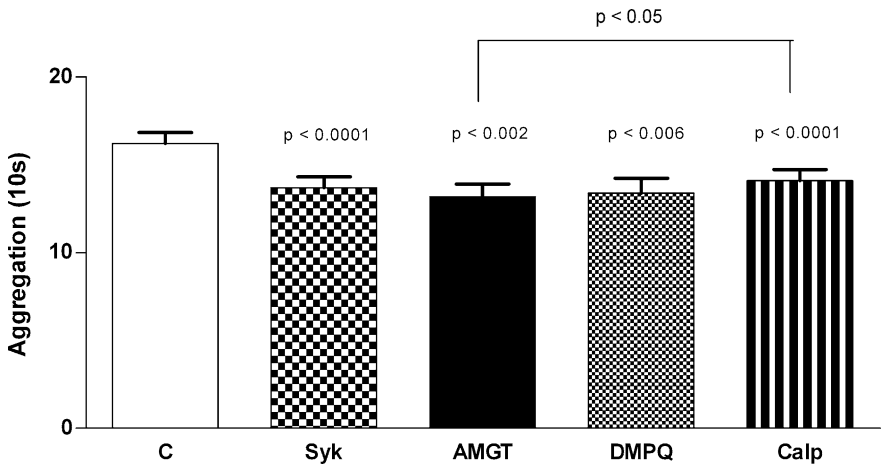

Fig. 1. Erythrocyte aggregation values (mean $\pm$ SD) obtained *in vitro* with protein tyrosine-kinases inhibitors (Syk, AMGT and DMPQ) and a phosphotyrosine-phosphatase inhibitor (Calp). There are significant decreases in the aggregation indexes with all the used inhibitors although a significant difference ($p < 0.04$) is observed between the totally phosphorylated (Calp) and the partially desphosphorylated (Syk, AMGT and DMPQ) state.

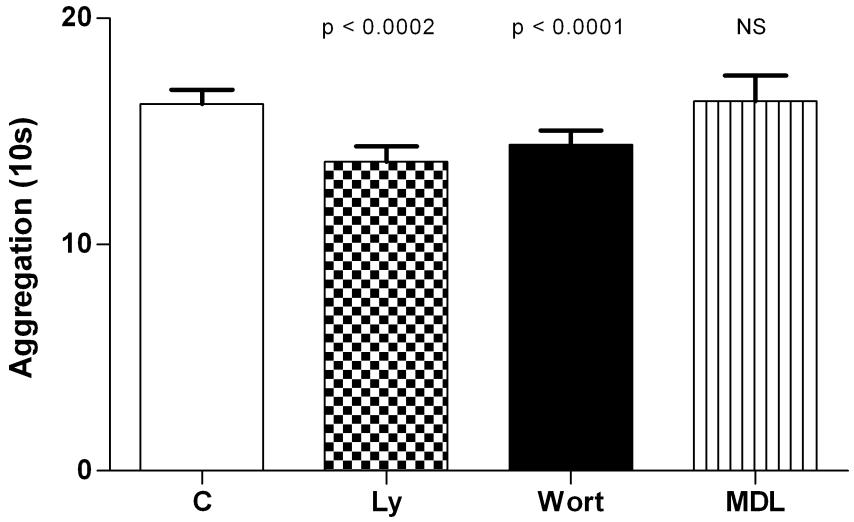

Fig. 2. Erythrocyte aggregation (mean ± SD) values after incubation with soluble guanylate cyclase inhibitor (Ly), PI3-K inhibitor (Wort) and adenylyl cyclase inhibitor (MDL).

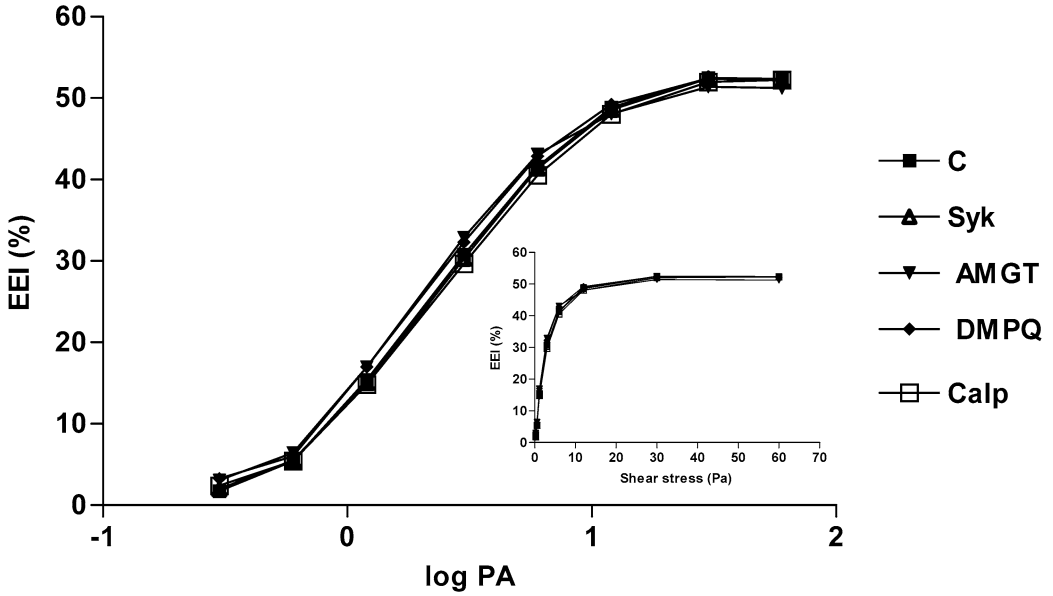

Fig. 3. Erythrocyte elongation index (EEI) with protein tyrosine-kinases inhibitors (Syk, AMGT and DMPQ) and phospotyrosine-phosphatase inhibitor (Calp). There were no significant differences on EEI.

3.3. Effects on pH, Hb, oxyHb and MetHb concentrations

Table 1 show that no significant differences are observed in pH, Hb and oxyHb concentrations values after the addition of the inhibitors to the blood samples aliquots. Methehemoglobin levels are, comparing with the control aliquot (1.5 ± 0.2%), increased (3.0 ± 0.5%) in the presence of a guanylate cyclase inhibitor (Ly).

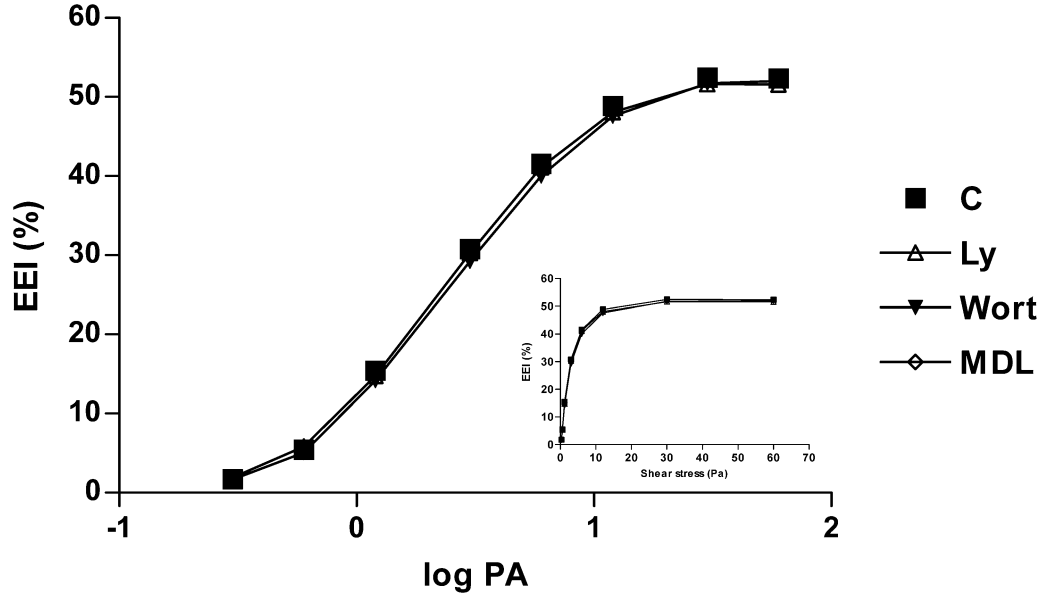

Fig. 4. Erythrocyte elongation index (EEI) obtained *in vitro* with guanylate cyclase inhibitor (Ly), PI3-K inhibitor (Wort) and adenylyl cyclase inhibitor (MDL). There were no significant differences on EEI.

Table 1

Values (mean ± SD) of whole blood concentrations of Na^+, Ca^{2+} and HCO_3^-, pH, and hemoglobin, oxyhemoglobin and methemoglobin concentrations after *in vitro* incubations with the studied inhibitors

	C	Syk	AMGT	DMPQ
[Na^+] (mmol/l)	139.4 ± 1.7	141.0 ± 2.3	142.1 ± 2.1	137.9 ± 1.5
[Ca^{2+}] (mmol/l)	1.0 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	1.0 ± 0.2
[HCO_3^-] (mmol/l)	25.5 ± 1.7	25.8 ± 1.2	25.18 ± 1.2	24.8 ± 1.1
pH	7.4 ± 0.03	7.4 ± 0.04	7.4 ± 0.02	7.4 ± 0.02
Hb (g/dL)	15.2 ± 1.5	15.2 ± 0.9	15.1 ± 1.3	15.6 ± 1.4
O_2Hb (%)	70.0 ± 11.2	67.3 ± 12.3	72.13 ± 11.1	72.18 ± 10.9
MetHb (%)	1.5 ± 0.2	1.6 ± 0.1	1.6 ± 0.1	1.5 ± 0.1
p50 (mmHg)	24.8 ± 1.9	26.0 ± 1.7	26.4 ± 1.6	23.0 ± 7.7
	Calp	Ly	Wort	MDL
[Na^+] (mmol/l)	142.0 ± 2.4	141.5 ± 1.8	142.7 ± 2.3	138.7 ± 1.5
[Ca^{2+}] (mmol/l)	1.0 ± 0.2	1.1 ± 0.2	1.0 ± 0.2	1.0 ± 0.2
[HCO_3^-] (mmol/l)	24.9 ± 2.9	25.7 ± 1.2	25.6 ± 1.8	23.4 ± 3.8
pH	7.4 ± 0.03	7.4 ± 0.03	7.4 ± 0.03	7.4 ± 0.02
Hb (g/dl)	15.4 ± 1.2	15.2 ± 1.1	15.1 ± 1.2	15.3 ± 1.0
O_2Hb (%)	65.5 ± 15.2	65.2 ± 12.5	67.3 ± 11.6	65.6 ± 17.9
MetHb (%)	1.5 ± 0.1	3.0 ± 0.5*	1.5 ± 0.1	1.5 ± 0.1
p50 (mmHg)	26.5 ± 1.7	25.8 ± 1.9	26.5 ± 1.7	26.0 ± 1.9

Syk, AMGT and DMPQ – PTK inhibitors; Calp – PTP inhibitor; Ly – Guanylate cyclase inhibitor; MDL – adenylyl cyclase inhibitor. Wort – PI3K inhibitor.

*$p < 0.0001$.

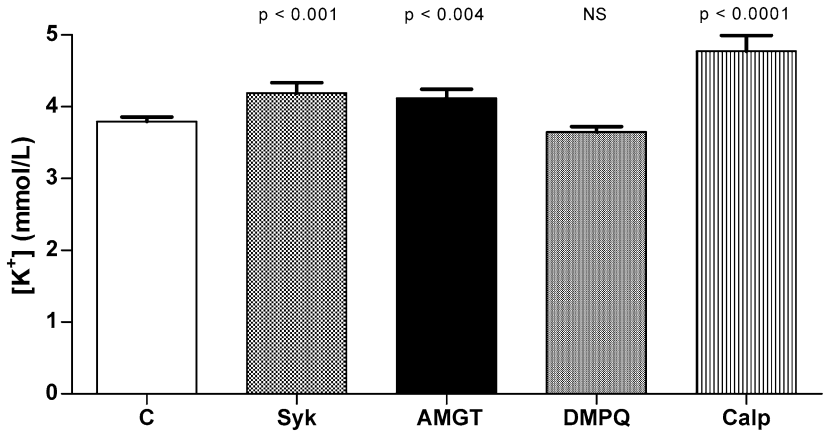

Fig. 5. Values (mean $\pm$ SD) of K^+ concentrations obtained *in vitro* with protein tyrosine-kinases inhibitors (Syk, AMGT and DMPQ) and phosphotyrosine-phosphatase inhibitor (Calp). There was a significant increase in the K^+ concentration with the PTK inhibitors Syk and AMGT, $p < 0.001$ and $p < 0.0007$, respectively. A highest increase is observed with the PTP inhibitor (Calp) $p < 0.0001$.

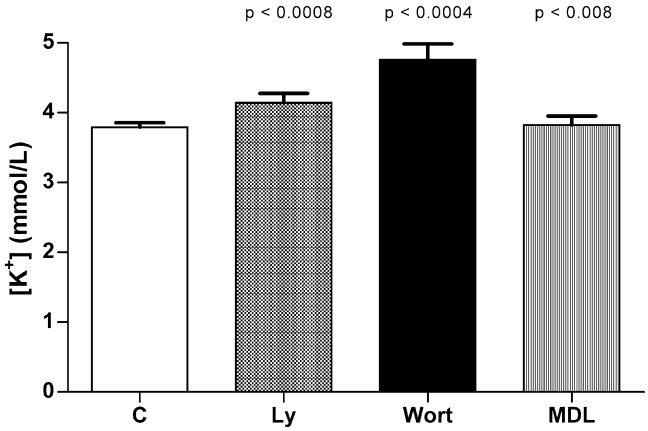

Fig. 6. Values (mean $\pm$ SD) of K^+ concentrations obtained *in vitro* with guanylate cyclase inhibitor (Ly), PI3-K inhibitor (Wort) and adenylyl cyclase inhibitor (MDL). PI3-K inhibitor significantly increases K^+ levels, $p < 0.004$.

4. Discussion

It is described that a *post*-translational modifications exist in the cytoplasmic domain of band 3 (cdb3) in the tyrosine residues under phosphorylation and dephosphorylation by PTKs (protein tyrosine kinase) and PTPs (protein-phosphotyrosine phosphatases) respectively [21,27]. In mature erythrocytes tyrosine kinases Syk (p72 syk) and Lyn (p56/53 lyn) a member of the c-src kinases family are responsible for phosphorylation states and a certain dynamic balance exist between these state and a dephosphorylation one mediated by PTB1B [2,20]. When cdb3 is phosphorylated the glycolytic enzymes (aldolase, phosphofrutokinase 1, glyceraldehyde-3-phosphate dehydrogenase) actually binding dissociated to the cytoplasm [29]. At variance when cdb3 is dephosphorylated those glycolytic enzymes are bound with a concomitant impairment of the lactic fermentation [15,43].

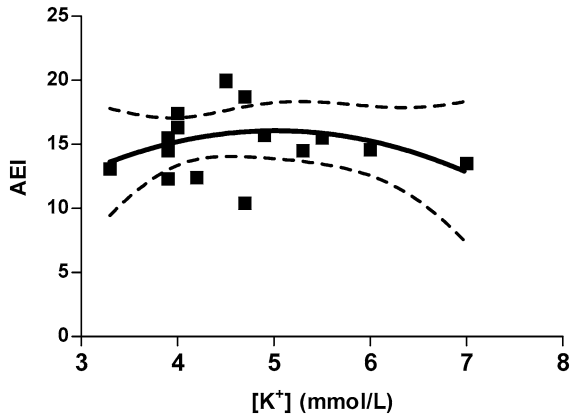

Fig. 7. Correlation between the erythrocyte aggregation indexes (EAI) and the potassium concentration in presence of the PI3-K inhibitor.

In this study was verified that the alteration on red blood cell protein band 3 phosphorylation and dephosphorylation states mediated by the PTK and PTP inhibitors induce a significantly decreased in erythrocyte aggregation and less unmodified erythrocyte deformability of venous blood samples collected from healthy humans (Figs 1–4). However the EAI values obtained in presence of Syk inhibitor are significantly lower than in presence of the PTP inhibitor (Fig. 1).

When calpeptin (PTP1B inhibitor) is added to the blood samples aliquots the band 3 became phosphorylated and an over stimulation of the lactic fermentation will be expected with the adenosine triphosphate produced (ATP) deviated by PTKs as its co-substrate. If it happens probably less amount of ATP is disposal to the physiological transport action of the Na^+/K^+-ATPase activity and in consequence an unbalance in the RBC ionic gradients could be expected. In fact, an increased in the plasma K^+ ions concentrations was observed in the blood samples aliquots in the presence of calpeptin (Fig. 5) which is in accordance with the above raised hypothesis, without any concentration dependence (Fig. 7).

However when the Syk and the Lyn inhibitors are added to the blood samples aliquots the cdb3 stays in a less phosphorylated state which comprise a great glycolytic enzyme affinity to the protein band 3, may be followed by a decrease in the lactic fermentation rate and an increase in the oxidative part of the pentose phosphate pathway as observed by [13]. Besides a reduced ATP concentration is expected, in relation to normal conditions, the remaining will be utilised in the active transport proteins Na^+/K^+-ATPase and Ca^{2+}-ATPase because no changes are observed in these ions plasma concentrations. These results could indicate that the observed values of erythrocyte aggregation are not dependent of the intracellular calcium concentration which has been described early by Cicco et al. as an activator of erythrocyte aggregation [9].

Considering our "*in vitro* model" for the study of the two states of protein band 3 a less phosphorylate and a more phosphorylate one and maintaining fibrinogen concentration constant, changes in the EAI are obtained with a significantly increase when cdb3 is more phosphorylated than it is dephosphorylated. This behaviour when compared with the control aliquot shows that both states decreases erythrocyte aggregation which seems not to be an ion concentration based dependent mechanism as discussed above. The signal transduction mechanism responsible for the EAI changes is under study.

The erythrocyte deformability values obtained at all shear stress tested with the ektacytometer were maintain unchanged and in an independent way of either the phosphorylated/dephosphorylated state of the protein band 3 (Fig. 3) or the plasma ion level. To verify any RBC deformability change reper-

cussions after the cdb3 phosphorylation/dephosphorylation it will be necessary to obtain either a decreasing /rupture in the number of membrane-skeleton bindings or absence of ankyrin as observed by others [7].

In the present work the values of erythrocyte deformability are also maintained unchanged when the inhibitors of the adenylyl cyclase (MDL), the guanylate cyclase (Ly) and the PI3K (Wort) are added to the erythrocyte blood aliquots (Fig. 4) and as a net result no changes affected by oscillations in plasma K^+ levels were observed (slight significant increase values in relation to the control aliquot but without physiological repercussions) (Fig. 6). In a similar way it may be admitted that these molecules stabilise the erythrocyte membrane when compared with the blood sample aliquot control. Bor-Kucukatay et al. [22] when utilise the selective inhibitor of the nitric oxide sensitive guanylate cyclase found a decrease on the erythrocyte deformability which could add some insight to our results in a way that the absence of RBC deformability changes obtained in presence of Ly may result either from an independent nitric oxide influence or other complementary not yet described mechanism generated by the decrease cGMP concentrations.

All together the inhibitors used in the present work do not interfere with any internal chain reactions susceptibility to phosphorylate the spectrin proteins of the cytoskeleton that is known to decrease the erythrocyte deformability [40].

At variance when either Ly or Wort were added, at fibrinogen concentration constant, the erythrocyte aggregation values significantly decreased (Fig. 2) whereas stay unchanged in presence of the adenylyl cyclase inhibitor, MDL. Others authors have verified a regulation of cyclic AMP phosphodiesterase activity by PTK and PTP [9] which could explain the absence of a net effect on erythrocyte aggregation. Concerning the action of Ly, a guanylate cyclase inhibitor, in RBC an increase of the MetHb concentration (Table 1) was obtained as a consequence of the absence of guanylate cyclase activation by NO. So it will expect that more NO is available to react with the oxygenated haemoglobin molecules originating MetHb and nitrites.

The inhibition of guanylate cyclase promotes a decrease in the cGMP concentration that could remove its "internal cross-talk" with the cAMP specific phosphodiesterase removing the inhibition of the respective enzyme activity, as has been verified by others authors, conducting to the intracellular decrease of the cAMP [30]. This could abolish the influence of the decrease of the cAMP concentration on EAI values because these were unchanged in the presence of MDL. So the decrease of EAI values obtained when Ly was added could occur by a signal transduction mechanism without the cAMP participation.

The significant $p < 0.0001$ decreased values of erythrocyte aggregation obtained in presence of Wort, the PI3K inhibitor (Fig. 2), was not associated with an increase of plasma potassium ion concentration which explanation need further studies such as testing the inhibitors utilised for different K^+ membrane transports systems [10]. It will need also to be verified if there is dependence from the increase of the lactic fermentation rate as a consequence of the lack of pyruvate kinase phosphorylation PI3K dependent [23].

In conclusion our results show that the erythrocyte aggregation tendency is higher when band 3 is phosphorylated than when it's partly desphosphorylated besides both band 3 states have lower EAI values in relation to the control blood values samples aliquots as a consequence of an *in vitro* manipulation by PTK and PTP inhibitors.

Other proteins phosphorylation targets of GC and PI3K will not be excluded as contributing to lower erythrocyte aggregation values.

It will be physiological relevant to test those inhibitors in blood samples with hyperviscosity syndromes.

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